SUPPLEMENTAL MATERIAL

Data S1

Supplemental Methods

Sample Collection

Human peripheral arteries (Table S1) were obtained from limb amputation surgeries (Emory University IRB #51432 and 70813) for participants either diagnosed with PAD or undergoing amputation for an unrelated reason, from a total of 14 donors (Table S1). No exclusion criteria were applied. Due to the difficulty in obtaining peripheral vessels, all available PAD and control vessels available were used. Samples were snap frozen in liquid nitrogen and stored in -80°C until shipped to Brigham and Woman's Hospital for downstream analysis. Samples were then dissected on ice, with half used for proteomics and the other half for histology. Tissue samples were placed in 200uL of ice-cold RIPA buffer supplemented with protease and phosphatase inhibitor (Pierce, USA) for subsequent homogenization using a Precellys tissue homogenizer (Precelly lysing kit, Precellys, USA) using 3x 10 second cycles of 5000 RPM settings. Homogenates were then centrifuged (max. speed, 10 mins, 4°C) and the supernatant used for downstream analysis. Serum samples were collected from PAD patients prior to surgery (Table S2). Control blood was purchased from 6 donors (Research Blood Components, USA). Serum was collected in BD vacutainer clot activator (BD#367812) tubes and stored at -80°C prior to downstream analysis.

Histological Analysis

PAD tissue was embedded in OCT tissue freezing medium and frozen. 7 um fresh-frozen sections were cut via cryostat and fixed depending on stain. Sections for Oil Red O staining were fixed in 10% formalin for 5 minutes, before washing 3x in distilled water. Following air drying, slides were placed in absolute propylene glycol for 5 minutes and stained with pre-warmed Oil Red O solution for 10 minutes at 60°C. Sections were differentiated in 85% propylene glycol solution for 5 minutes before rinsing 2x in distilled water. Sections were then counter-stained in Mayer's haematoxylin for 30 seconds and washed in water before mounting with aqueous mounting media. Sections for

Alizarin red staining were fixed in ice-cold acetone (100%) for 10 minutes at room temperature, before washing twice in PBS for 5 minutes. Slides were then submerged in 2% Alizarin red stain for 1 minute room temperature, before incubating with acetone (1 min), 50:50 acetone:xylene (1 min), xylene (1 min) and mounting with xylene-based mounting media. Sections were fixed in 4% PFA for 5 minutes, before haematoxylin and eosin staining conducted as previously described [9]. For immunohistochemical analysis sections were blocked with 0.3% hydrogen peroxidase (Fisher Scientific, USA) and incubated with 4% horse serum (Dako, USA). Primary antibodies (Table S3) were diluted in 4% horse serum and incubated for 90 minutes at room temperature in a humidified chamber and then incubated with biotinylated mouse or rabbit anti-goat secondary antibodies (Dako, USA). The streptavidin peroxidase method was used for each stain, and the reaction was visualised with a 3-amino-9-ethylcarbazol substrate (AEC substrate chromogen, Dako, USA). Sections were counter-stained with Gills Haematoxylin. All slides were imaged on a Nikon eclipse 50i.

Transmission Electron Microscopy

Fresh frozen sections were cut at 20 uM sections and placed in 4% PFA with 0.1% glutaraldehyde for 2 hours before placing in PBS supplemented with 20mM glycine and delivered to the Harvard Medical School Electron Microscopy Facility. Samples were then infiltrated with 2.3 M sucrose in PBS for 15 minutes and cut onto carbon coated copper grids before imaging by the core on an AMT camera system, HV=80.0kV, cal = 0.002 micron/pix.

Immunofluorescence

PAD sections were prepared as above. 7m fresh-frozen sections were cut via cryostat and fixed with 4% PFA for 10 minutes, washed 2x in PBS and blocked with 4% normal serum for 30 minutes. Sections were then incubated with primary antibodies (Table S3) for 1h at room temperature. Following washing 2x with PBS, sections were incubated with secondary antibodies for 30 minutes at room temperature. Sections were then washed as before and incubated with

DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride, Thermo, USA) for nuclear counter staining. Sections were imaged using confocal microscopy on a Nikon confocal A1 microscope.

NLRP3 ELISA

Human NLRP3 SimpleStep ELISA (ab27401, Abcam, USA) was run following manufacturer's instructions. Briefly, 50 uL of standards and 50 uL of 1:10 diluted tissue samples and 1:1 serum diluted samples were loaded in duplicate. Following 1 hour of incubation with 50 uL antibody cocktail all wells were washed 3x in wash buffer and developed with 100 uL of TMB development solution for 10 minutes. Finally, 100 uL of stop solution was then added and the plate read at OD 450nm on a spectramax 3 (Molecular probes, USA).

Tissue Proteolysis

Homogenized human peripheral vessels samples were proteolyzed using the iST in-solution digestion kit (PreOmics, USA) automated on the PreON robot (PreOmics, USA). Briefly, 50ug of protein was lysed with 50 uL LYSE buffer and heated to 95°C for 10 minutes. Samples were digested for 2 hours following manufacturer's instructions. Eluted peptides were then dried via speed vacuum (Eppendorf Vacufuge) and resuspended in 40 uL in LC-LOAD. 4 uL of 1:20 diluted peptide stock (250 ng protein stock) was analysed via mass spectrometry.

Mass Spectrometry

Data-dependent acquisition (DDA) mass spectrometry was acquired on the Orbitrap Fusion Lumos coupled to a heated EASY-spray nanosource and nanoLC1000 (Thermo Fisher Scientific, USA). Peptides were first subjected to an Acclaim PepMap RSLC C18 trap column and then separated with a heated EASY-Spray column (Thermo Fisher Scientific). The analytical gradient was run at 300 nl/min from 5 to 21% Solvent B (acetonitrile/0.1% formic acid) for 75 minutes, 21 to 30% Solvent B for 10 minutes, followed by 10 minutes of 95% Solvent B. Solvent A was water/0.1% formic acid. Acetonitrile and water were LC-MS-grade. Analytical gradient was run at 300 nl/min from 5 to 21% Solvent B (acetonitrile/0.1% formic acid) for 75 minutes, 21 to 30%

Solvent B for 10 minutes, followed by a 95% to 5% Solvent B jigsaw wash. Solvent A was water/0.1% formic acid. Acetonitrile and water were LC-MS-grade. The Orbitrap was set to 120 K resolution, and the top N precursor ions in 3 seconds cycle time within a scan range of m/z 400-1500 (60 sec dynamic exclusion) were subjected to collision induced dissociation (CID; collision energy, 30%; isolation window, 1.6 m/z). The ion trap was set to a rapid scan rate for peptide sequencing (MS/MS).

Mass Spectral Analysis

DDA spectra collected from the scan range of m/z 400-1500 were queried against the human UniProt database (downloaded September 2020; 96, 816 entries) with the HT-SEQUEST search algorithm, via Proteome Discover 2.2 (Thermo Fisher Scientific) using a 10 ppm tolerance window in MS1 and a 0.6 Da fragment tolerance window for MS2; trypsin as the protease with carbamidomethylation as a static modification and oxidation of methionine as variable modification. The peptide false discovery rate of 1% was calculated using Percolator within Proteome Discover (PD). Peptides assigned to a given protein group, and not present in any other protein group, were considered as unique. Consequently, each protein group is represented by a single master protein (PD Grouping feature). Unique and razor peptides were used per protein for quantification. Quantification of proteins across samples (the 14 vessel samples) was completed via Feature Mapper. Chromatographic alignment, the maximum retention shift was 10 minutes and mass tolerance of 10 ppm. Precursor peptide abundances were based on their chromatographic intensities and total peptide amount was used for normalization. Proteins used for downstream quantification were filtered by having 2 or more unique peptides. Magnitude of missing values was 10.69% of total proteins identified. The final protein list is therefore a consensus proteome of the 14 vessels. Proteome Discover results were normalised by the protein median before downstream analysis via an inhouse script.

NLRP3 Targeted Mass Spectrometry

1 ug NLRP3 (H00114548, Novus Biologicals, USA) was proteolyzed as above and analysed by the Lumos using a shorter analytical gradient: 300 nl/min from 8 to 25% Solvent B for 20 minutes, 25 to 35% Solvent B for 5 minutes, followed by the jigsaw wash. The Orbitrap was set to 120 K resolution, and the top N precursor ions in 3 seconds cycle time within a scan range of m/z 400-1500 (30 sec dynamic exclusion) were subjected to higher energy collision induced dissociation (HCD; collision energy, 30%; isolation window, 1.2 m/z; 30 K resolution) for MS/MS. The most abundant NLRP3 peptide (Table S5), as identified using Proteome Discoverer, was monitored in the 14 vessel samples. The peptide was monitored using the targeted MS2 module acquired in profile mode: precursor isolation window, 0.8 m/z; collision energy, 30%, scan range, m/z 350-1200; resolution, 240 K. The extracted ion chromatogram of each fragment ion's monoisotopic peak was quantified using QualBrowser (Thermo Fisher Scientific).

Differential Enrichment and Gene Ontology Analysis

Differentially abundant proteins between PAD and control were identified using *limma* 3.46 [10] in R using whole tissue proteomics. Briefly, limma uses lmFit() and eBayes() to estimate the variance by exchanging information between genes for reducing false positives and detect genes with large variance (logFC<2 and adjusted p-value (Benjamini-Hochberg) < 0.05). The differential abundant proteins list was then analysed for enriched pathways using clusterProfiler 3.18 [11] in R using function enrichGO. The ontologies were plotted using cnetplot().

Multi-Analyte LegendPlex Flow Assay

Human Inflammation panel 1 and a custom LegendPlex panel (containing IL-6, IL-18, IL-9 and IL-1β) (Cat. 740809 & 900001042 Biolegend, USA) was conducted following manufacturer's instructions. Briefly, serum samples were diluted 2-fold with assay buffer before use. 25 uL of diluted sample was incubated with 25 uL of assay buffer and 25 uL of standards were incubated with 25 uL Matrix B3 and B1 respectively. 25 uL of human inflammation panel 1 premixed beads were added and the plate was shaken at 800 rpm for 2 hours at room temperature. The plate was then centrifuged and supernatant removed and the beads were washed twice with 200uL 1x wash

buffer. 25 uL of detection antibodies were then added and shaken at 800 rpm for 1 hour at room temperature. Subsequently 25 uL of Streptavidin, R-Phycoerythrin (SA-PE) was added directly to each well and shaken at 800 rpm for 30 minutes at room temperature. Wells were then washed again as before, and beads resuspended in 150uL wash buffer and assessed immediately on the Cytek Aurora, (Cytek, USA).

Statistical Analysis

Graphpad Prism (V9.0) and R (V4.2) was used to analyse data. Non-parametric tests such as Wilcoxon test were conducted on non-normally distributed data. Association of NLRP3 and cytokines was conducted via linear regression on Graphpad Prism. Graphs were produced in Prism and R. Proteomic data exploration was conducted using Qlucore Omics Explorer 3.2 (Qlucore, Sweden).

Table S1. Patient Tissue Demographics.

ID	PAD	Age	Sex	Diabetes	Dyslipidemia	Smoking	Chronic F	Indication	Sample
#2	Y		60 M	Y	N	N	N	Infection	Peroneal artery
#3	Y		32 M	N	N	N	Y	Infection	Tibial artery
#4	Y		76 M	N	Y	Y	N	Ischemic	Popliteal artery
#5	Y		76 F	Y	N	N	N	Infection	Peroneal artery
#8	Y		67 F	Y	Y	N	N	Ischemic	Tibial artery
#9	Y		46 M	N	N	N	N	Ischemic	Popliteal artery
#12	Y		81 F	N	N	N	N	Ischemic	Tibial artery
#19	Y		66 M	Y	N	N	Y	Ischemic	Peroneal artery
#28	Y		66 M	Y	N	N	Y	Infection	Tibial artery
#29	Y		59 M	Y	N	N	N	Ischemic	Peroneal artery
#33	N		29 M	N	N	N	N	Control	Tibial artery
#77	N		67 F	Y	N	N	N	Control	Tibial artery
#107	N		67 M	N	N	N	N	Control	Peroneal artery
#108	N		74 M	N	N	N	N	control	Peroneal artery

Table S2. Patient serum demographics.

ID	PAD	Age	Sex	Diabetes	Dyslipidemia	Smoking	Chronic Kidney Disease	Indication	Sample
01-0043	Y	43	M	Y	N	N	Y, ESRD	PAD	Serum
01-0047	Y	77	M	Y	N	N	N	PAD	Serum
01-0051	Y	75	F	N	Y	N	N	PAD	Serum
01-0065	Y	70	M	N	Y	Y	N	PAD	Serum
01-0066	Y	62	M	Y	Y	N	N	PAD	Serum
01-0070	Y	68	M	N	N	N	N	PAD	Serum
01-0072	Y	64	F	Y	N	Y	N	PAD	Serum
01-0080	Y	54	F	N	N	N	N	PAD	Serum
1-64379	N	55	F	N	N	N	N	Control	Serum
2-64373	N	38	M	N	N	N	N	Control	Serum
3-64372	N	55	M	N	N	N	N	Control	Serum
4-64375	N	34	M	N	N	N	N	Control	Serum
5-64377	N	45	M	N	N	N	N	Control	Serum
6-64374	N	56	M	N	N	N	N	Control	Serum

Table S3. Antibodies Used.

Epitope	Supplier	Catalog No.	Dilution	Use
CD68	Abcam	ab213363	1:200	IHC
CD209	Abcam	Ab245200	1:200	IHC
NLRP3	Novus Biologics	NBP2-12446	1:100	IHC
Caspase 1	Cell Signalling Technology	D57A2	1:500	IHC
IL-1 beta	Cell Signalling Technology	12703	1:500	IHC
NLRP3	Novus Biologics	NBP2-12446	1:200	ICC
CD68	Abcam	ab213363	1:500	ICC

Table S4. NLRP3 Peptide Library.

NLRP3 peptide	RT start (min)	RT end (min)	Precursor m/z	Precursor charge	Fragment ion	Fragment ion m/z
GDILLSSLIR	22.5	26	543.83	2	y6	688.435
GDILLSSLIR	22.5	26	543.83	2	y5	575.351
GDILLSSLIR	22.5	26	543.83	2	y7	801.516

Table S5. Gene Ontology Input for Networks. See Excel file.